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# Electric currents in *Xenopus* tadpole tail regeneration

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ABSTRACT

*Xenopus laevis* tadpoles can regenerate tail, including spinal cord, after partial amputation, but lose this ability during a specific period around stage 45. They regain this ability after stage 45. What happens during this "refractory period" might hold the key to spinal cord regeneration. We hypothesize that electric currents at amputated stumps play significant roles in tail regeneration. We measured electric current at tail stumps following amputation at different developmental stages. Amputation induced large outward currents leaving the stump. In regenerating stumps of stage 40 tadpoles, a remarkable reversal of the current direction occurred around 12–24 h post-amputation, while non-regenerating stumps of stage 45 tadpole maintained outward currents. This reversal of electric current at tail stumps correlates with whether tails regenerate or not (regenerating stage 40—inward current; non-regenerating stage 45—outward current). Reduction of tail stump current using sodium-free solution decreased the rate of regeneration and percentage regeneration. Fin punch wounds healed normally at stages 45 and 48, and in sodium-free solution, suggesting that the absence of tail re-growth at stage 45 is regeneration-specific rather than a general inhibition of wound healing. These data suggest that electric signals might be one of the key players regulating regeneration.


## Introduction

The tadpole of the frog *Xenopus laevis* has the ability to regenerate a complete tail (including spinal cord, muscle, notochord, etc.) following partial tail amputation. It is therefore an excellent model system for investigating tissue repair and regeneration (Slack et al., 2008; Tseng and Levin, 2008; Taniguchi et al., 2008). Recent research has provided significant insights into a range of fascinating cellular and molecular mechanisms controlling how a damaged or amputated appendage repairs and re-grows complex tissues and structures. Wnt-FGF signaling, TGF beta, bone morphogenic protein and Notch signaling are all required for tail regeneration (Beck et al., 2003; Ho and Whitman, 2008; Lin and Slack, 2008; Kragl et al., 2009).

In *Xenopus* tadpoles, tail regeneration occurs throughout development, except for a "refractory period" between stages 45 and 47 (4–6 days of development), when the tail heals over without regeneration (Beck et al., 2003). Thus, the tadpole tail does not regenerate during this specific period, but it can do so both before and after this stage. This suggests that there are critical "regeneration factors" that are missing during the refractory period. Regeneration can be enabled during this refractory period by activation of either the bone morphogenic protein or Notch signaling pathways (Beck et al., 2003). Bone morphogenic protein causes regeneration of all tissues, whereas Notch signaling activates regeneration of spinal cord and notochord, but not muscle. Regenerative capability can therefore be enabled by genetic modifications that reactivate specific components of the developmental program.

Alongside these exciting discoveries of genetic and chemical control of regeneration, a less well-studied signal–electric fields generated by ion flow–emerges as a powerful signaling mechanism for tadpole tail regeneration and wound healing (Zhao et al., 2006; Adams et al., 2007). In cells, tissues, organs, and whole organisms, injuries induce ion flow and long-lasting endogenous voltage gradients that regulate wound healing and regeneration (Forrester et al, 2007; McCaig et al, 2005; Reid et al., 2005). Surprisingly, an applied electric field of physiological strength has an overriding effect to direct cell migration in wound healing (Zhao et al., 2006). Electrical stimulation can enhance anatomical and behavioral recovery after spinal cord hemisection in guinea pigs and following accidental paraplegia in dogs (Borgens et al., 1987). These results led to an ongoing human clinical trial in which battery implants are being used to treat spinal cord injury (http://www.vet.purdue.edu/cpr/). A molecular link between biophysical events and regeneration has recently been uncovered. Activity of the V-ATPase $H^+$ pump is required for regeneration, but not wound healing. Crucially, induction of $H^+$ flux is sufficient to rescue axonal patterning and tadpole tail outgrowth in otherwise non-regenerative conditions (Slack, 2007; Adams et al., 2007). The electric currents at amputated *Xenopus* tail stumps, however, have not been measured until now.

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Using a vibrating probe system (Jaffe and Nuccitelli, 1974; Reid et al., 2007), we demonstrate that a signature electric current flow directly predicts regeneration of tadpole tails. Manipulation of the current significantly altered regenerative ability. Electric currents at the stump may therefore be a key "regeneration factor" in tail/spinal cord regeneration that influences or acts in parallel with BMP-Notch-mediated regeneration.

## Materials and methods

### *Tadpoles*

Tadpoles (*X. laevis*) were kindly supplied by Dr. Stefan Hoppler (Department of Biomedical Sciences, University of Aberdeen, Scotland, UK), and also obtained from Xenopus Express (www.xenopus.com). Tadpole stages were identified by reference to Nieuwkoop and Faber's normal table of *X. laevis* (Nieuwkoop and Faber, 1994). Tadpoles were maintained in Marc's modified Ringer (MMR) which contained (mM): 10 NaCl; 0.2 $CaCl_2$; 0.2 KCl; 0.1 $MgCl_2$; 0.5 HEPES (pH 7.4) (Sigma-Aldrich, St. Louis, MO). Tadpoles were incubated at 16 °C until the desired stage was reached. Prior to fluorescent imaging, cutting or probe measurements, tadpoles were anesthetised in 1 mM pharmaceutical grade tricaine methanesulfonate (trade name Finquel), buffered to pH 7–7.4 (Argent Chemical Laboratories, Inc.). After anesthesia, tadpoles were placed in normal MMR and recovered in 5–10 min. Tails were cut using fine spring scissors (Fine Science Tools), and approximately half of the tail was removed (see Fig. 1A).

In experiments where we compare tail current and regeneration, different groups of tadpoles were used. For example, one group of 88 tadpoles (from 4 different spawnings or batches) provided the "normal" stage 40 percentage regeneration data (Figs. 1, 5), whereas a different group of tadpoles were used for the stump current measurements (Figs. 4, 5). Thus, we did not measure individual tadpoles then monitor the same tadpoles for regeneration, but used different groups to expedite the process. The total number of individual tadpoles, and the number of spawnings or batches, per experimental group are given in the text or figure legends where appropriate.

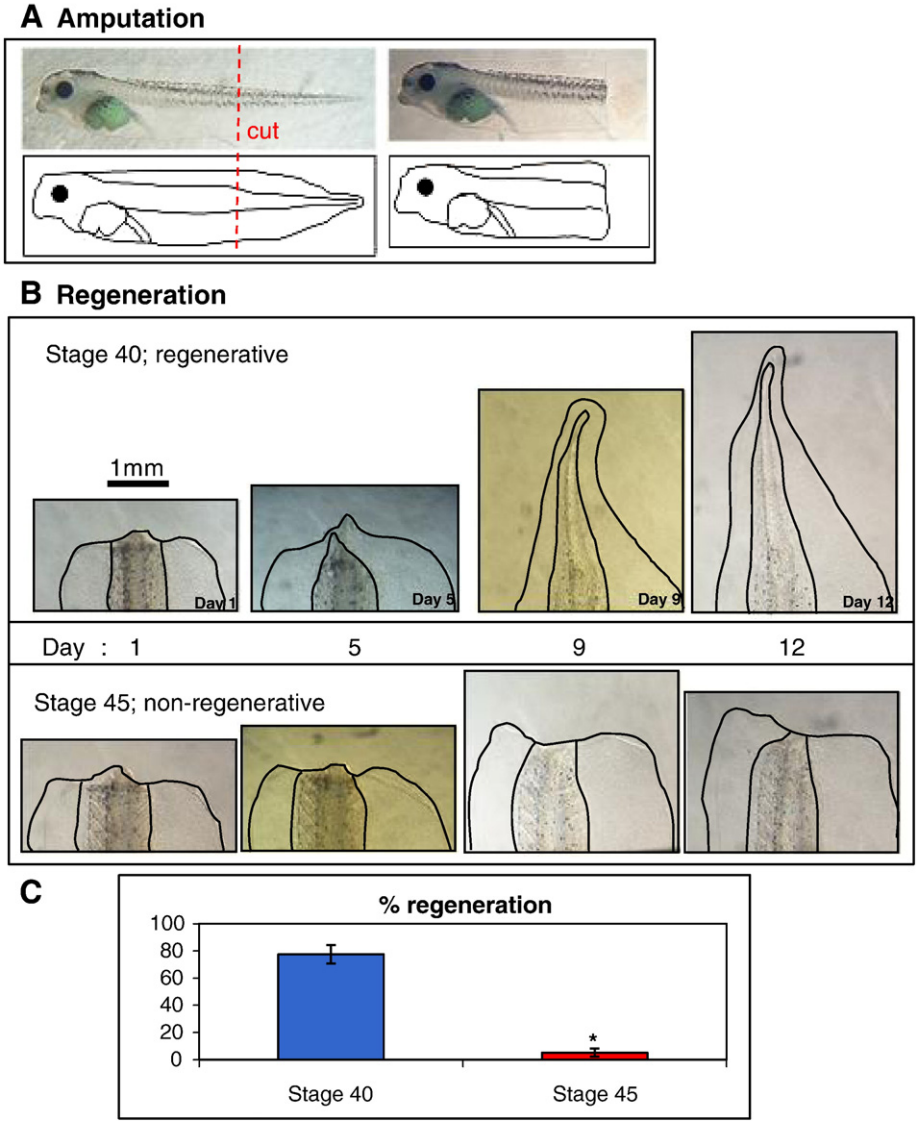


**Fig. 1.** Tadpoles lose the ability to regenerate tails at stage 45. (A) For regeneration experiments and probe measurements approximately 50% of the tail was amputated. (B) This montage compares a single tail cut at stage 40 with another cut at stage 45. The stage 40 tail begins to re-grow about 5 days after cutting, and is almost fully regenerated at 12 days. The stage 45 tail does not re-grow. Scale bar 1 mm. (C) Percentage regeneration was dramatically reduced at stage 45. More than three quarters of stage 40 tails regenerated (77.6%) whereas only 5.2% of stage 45 tails re-grew. Data from 4 different spawnings (batches) of tadpoles; total numbers: 88 (stage 40), 76 (stage 45); *$P<0.001$.

*Tail regeneration analysis*

To measure regeneration rate and percentage regeneration, tadpoles were photographed immediately after cutting and then again after 7, 10 and 12 days. Comparison of "before" and "after" pictures allowed scoring of regeneration or non-regeneration in different stages or treatments, and calculation of percentage regeneration. Regeneration rates were determined by measuring, from photographs, the length of tail that regenerated in 10 days using the ImageJ (http://rsbweb.nih.gov/ij/) measuring tool, and are presented in arbitrary units (AU).

*Fluorescent imaging*

For fluorescent imaging, tails were amputated and the tadpoles kept for 5–7 days at 16 °C to allow time for tail regeneration to begin, prior to labeling.

*GAP43 (growth associated protein 43) spinal cord label*

Tadpoles were fixed in 4% paraformaldehyde in MMR for 2 h then washed in MMR. To label with primary antibody, tadpoles were incubated at room temperature in MMR containing mouse anti-GAP43 antibody (Sigma-Aldrich; 1/500) with 0.5% triton X-100 (Sigma-Aldrich), for 18 h. Tadpoles were washed thoroughly in MMR and then incubated in Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes; 1/500) for 4 h and then washed overnight in MMR.

*DiI microinjection*

Tadpoles were first anesthetized as above. DiI-Ac-LDL (DiI complexed with acetylated low-density lipoprotein; Molecular Probes) was injected into the beating hearts using an Eppendorf Transjector 5246 microinjection system with an Eppendorf sterile femptotip mounted on a micro-manipulator (see Fig. 2B). Tadpoles were removed from the anesthetic and placed in MMR for recovery. They were incubated overnight at 16 °C and anesthetized again prior to imaging.

*DiBAC membrane potential label*

Tadpoles were incubated at room temperature in MMR containing 0.2 mM $DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid)-trimethine oxonol) (Molecular Probes) for 20 min and washed several times in MMR. They were then anesthetized prior to imaging.

Brightfield and fluorescent images were taken on a modified Nikon M2B microscope with ×40 water immersion lens and Hamamatsu Orca CCD camera controlled by Improvision OpenLab software running on an Apple Macintosh computer.

*Vibrating probes*

The vibrating probe measures net electrical current flow non-invasively. Preparation of vibrating microelectrodes in this laboratory has been described in detail previously (Reid et al., 2007). Prior to use, probes were calibrated in MMR (plus 1 mM tricaine anesthetic) by

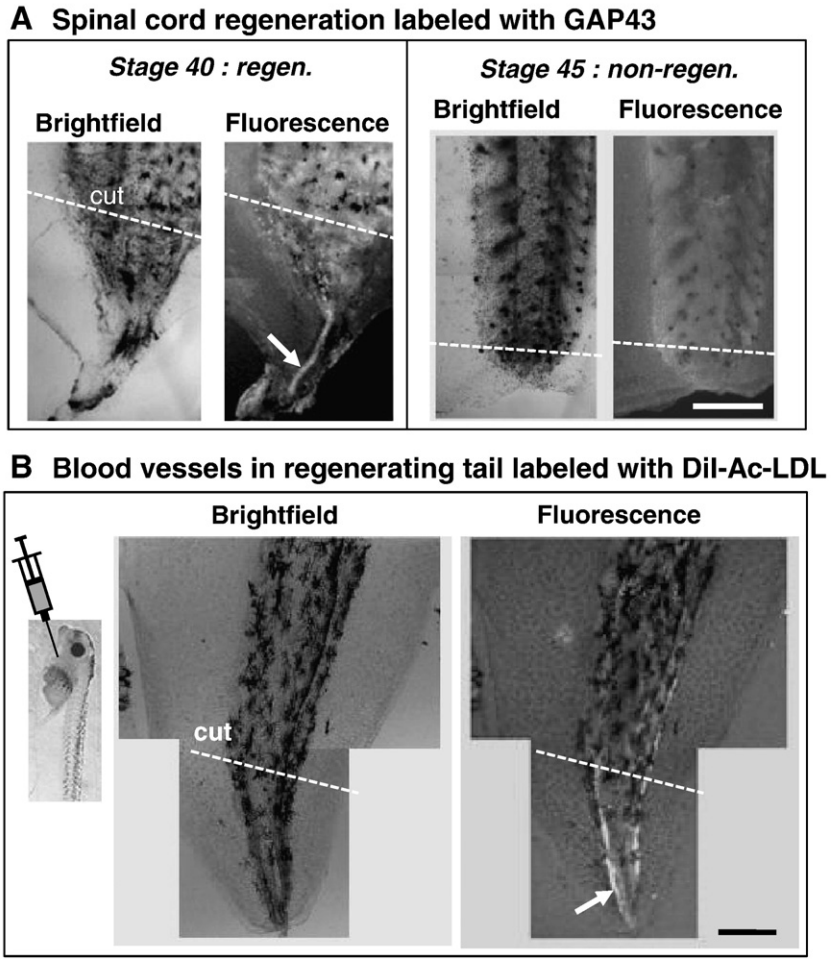


**Fig. 2.** Regenerating tails show active nerve and blood vessel growth. (A) Fluorescent imaging of spinal cord regeneration. GAP43 (growth associated protein 43) specifically labels new growth of nerve. GAP43 labeling 6 days after tail cutting reveals regeneration of spinal cord (arrow) at stage 40 but not stage 45. (B) Labeling of sprouting blood vessels by fluorescent dye injection. DiI-Ac-LDL was injected into the beating hearts of anesthetized tadpoles 7 days after tail amputation. Dye in the bloodstream is taken up by vascular endothelial cells, labeling sprouting blood vessels in regenerating tails (arrow). Scale bars 0.5 mm.

application of a current density of exactly 1.5 μA/cm$^2$. The probe was also calibrated at the end of experiments in used MMR (+tricaine) to account for evaporation. For vibrating probe measurements, tadpoles were mounted in custom-made chambers. These were 55 mm plastic dishes into which was glued a semi-circular section of 1 mm-thick plastic. Onto this was glued a length (~3 cm) of insulated stainless-steel wire with a V-shaped bend to hold the tadpole immobile during measurements and imaging (see Fig. 4A). We measured tail stump currents up to 12 days post-amputation. Measurements were made at different positions across the cut tail stump, corresponding to different anatomical regions, but were not significantly different (see Fig. 3). Consequently, to save time, measurements were made only at the spinal cord (position "b" in Fig. 3A, and see also Fig. 4B).

*Ion substitution and drug treatment*

Na-free solution contained (mM): 0.2 $CaCl_2 2H_2O$; 0.2 KCl; 0.1 $MgCl_2 6H_2O$; 0.5 HEPES; 10 Choline chloride. Cl-free solution contained (mM): 0.2 $Ca(NO_3)_2 4H_2O$; 0.2 KOH; 0.1 $MgSO_4 7H_2O$; 10 NaOH; 10.8 methanesulfonic acid; 0.5 HEPES. Na- and Cl-free solutions were adjusted to pH 7.4 prior to use.

Drugs were applied at the following concentrations: Aminophylline, 10 mM; ascorbic acid, 10 mM; ouabain, 2 mM; furosemide, 0.2 mM. In ion-substitution and drug-treatment experiments, tadpoles were kept in the appropriate solution (Na-free, Cl-free or drug) before and after tail cutting and up to the end of the experiment. In regeneration experiments lasting up to 14 days, tadpoles which died during the study were eliminated and not scored as "non-regenerators". Survival rates in ion-free or drug solutions were not significantly lower than in normal MMR solution (except for ascorbic acid, in which tadpoles died prematurely before regeneration could begin). At the end of experiments, tadpoles were euthanized by placing in a high concentration of anesthetic (10 mM tricaine) until the heartbeat stopped (usually in 20–30 min). All procedures were approved by the University of California, Davis, Institutional Animal Care and Use Committee (protocol #15241) and the Department of Fish and Game (detrimental species permit no. 537).

*Statistics*

Data are expressed as mean ± standard error of the mean (SEM). Differences between mean values were compared using a two-sample Student's *t* test, performed with equal or unequal variance according to an *f* test. In graphs, asterisks and other symbols (α, #) indicate significant difference ($P<0.05$).

## Results

*Stage-specific tail regeneration*

We first characterized the regeneration ability of tadpoles at different stages. Most tadpoles around stage 40 regenerated, forming an almost complete new tail in 12–14 days, whereas most stage 45 tadpoles showed no regrowth at all (Fig. 1B). Regeneration began around day 5. More than three quarters of stage 40 tadpoles regenerated (77.6 ± 8.3%), whereas only 5.2 ± 3% of stage 45 tadpoles were able to regenerate ($P<0.001$, Fig. 1C).

To confirm that regenerating tails were able to re-grow complex tissues and structures, we labeled spinal cord and vasculature in regenerating tails. We used antibodies against GAP43 (growth-associated protein) to label nerve, and DiI-Ac-LDL to label endothelial cells in blood vessels. GAP43 is a nervous tissue-specific membrane protein expressed at high levels in regenerating and growing nerve fibers. We labelled regenerating and non-regenerating tails with anti-GAP43 antibody 6 days after tail cutting. The regenerating spinal cord was visible in the growing tails of stage 40 tadpoles but not stage 45 (Fig. 2A).

DiI-Ac-LDL (fluorescent marker DiI complexed with acetylated low-density lipoprotein) specifically labels endothelial cells. When injected into beating tadpole hearts, DiI-Ac-LDL enters the circulation and is taken up by vascular endothelial cells that possess "scavenger" receptors specific for the modified LDL, thus labeling blood vessels. We were able to observe sprouting blood vessels in regenerating tails (Fig. 2B). Thus we confirm that regenerating tails can re-grow and reorganize complex tissues like blood vessels and spinal cord.

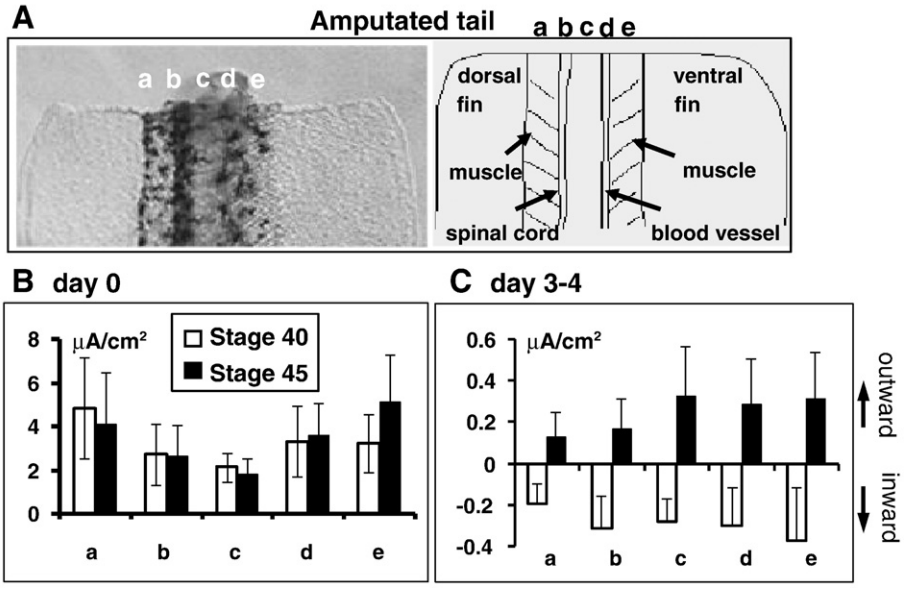


**Fig. 3.** Electric currents at stumps correlate with regeneration. Currents were measured at different positions on the cut tail stump. Consistently, the currents at all positions correlated with regeneration. (A) Photograph and schematic drawing showing the five measuring positions. They correspond to: dorsal fin muscle (a), spinal cord (b), muscle (c), blood vessel (d), ventral fin muscle (e). (B) Immediately after amputation (day 0), large outward currents (positive values) were detected at all positions. (C) At all positions, 3–4 days after cutting, stump currents in tadpoles of stage 45, which do not regenerate, remained outward . However, currents in stage 40 tadpoles reversed direction, becoming inward (negative values) at all positions of measurement. Data from 3 different batches of tadpoles; total numbers: 14 (stage 40), 16 (stage 45).

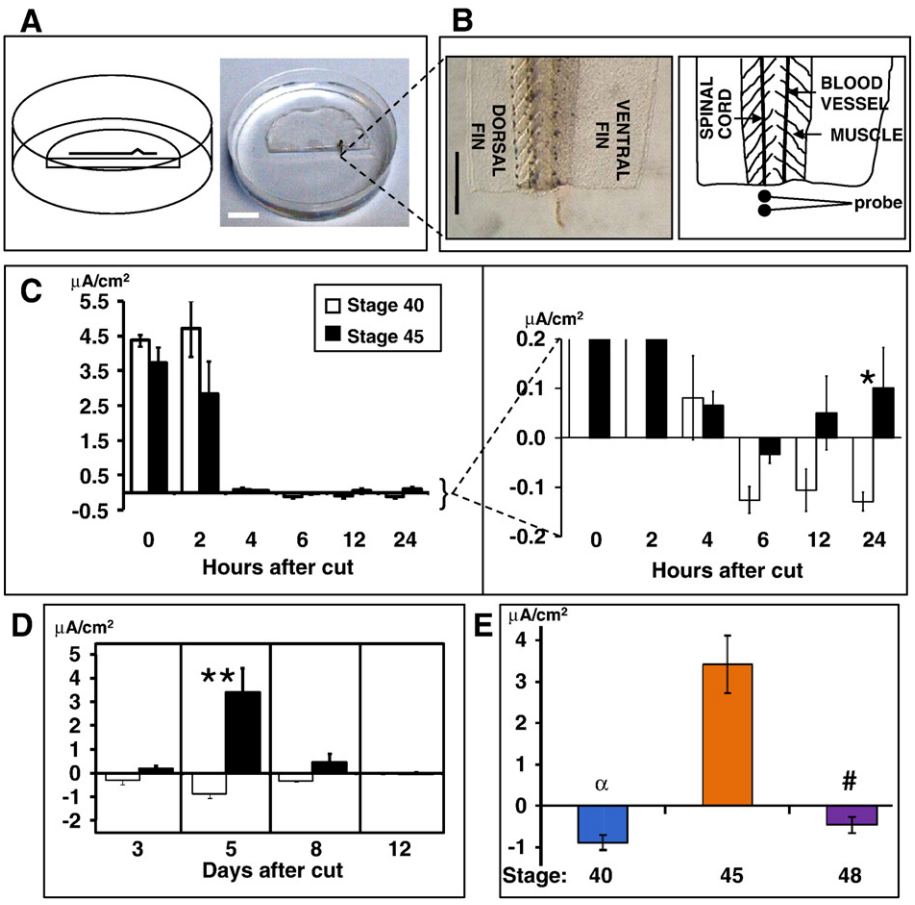


**Fig. 4.** Electric currents at stumps correlate with regeneration. (A) Chamber constructed to hold tadpoles for measurement and imaging (scale bar 1 cm). (B) Anatomy of tadpole tail. Measurements were made adjacent to the spinal cord. Scale bar 1 mm. (C, D) Tail stump current during regeneration, 0–24 h, and up to 12 days. Tadpole tail electric current correlates with tail regeneration. Immediately following amputation, large currents flow out of the stump in tadpoles of both stages 40 and 45. The currents then decreased. In regenerating tails, the direction of current reversed. Current at stage 40 and 45 tails was significantly different at 24 h after amputation (*$P<0.03$) and at the time that stage 40 tails begin to re-grow (day 5; **$P<0.01$). Data from 4 different batches of tadpoles; total numbers: 25 (stage 40), 18 (stage 45). (E) The inward currents at day 5 were confirmed in regenerating stage 40 and 48 tadpoles. Only non-regenerating stage 45 had outward currents. $^{\alpha}P<0.01$; $^{\#}P<0.02$. Stage 48 $n=18$ from 3 different batches.

*Electric current at tail stump predicts regeneration*

To establish the overall electric current profile of normal stages 40 and 45 tadpoles, we used a vibrating probe to measure current at various body positions in intact tadpoles. Anesthetized tadpoles were gently immobilized in a dish (see Fig. 4A). Intact tadpoles at both stages had small inward currents (negative values: −0.04 to −0.27 μA/cm$^2$; Table 1) at all body positions measured (head, back, flank, belly, side of tail) except for the gills on either side of the head which had a large outward current (positive value: 0.77 ± 0.04 μA/cm$^2$) ($n=10$ tadpoles of each stage from 3 different batches). Thus, the small current flowing into the tadpole surface (skin) is balanced by a large current flowing out at the gills, forming a loop of current flow.

To determine whether regenerating and non-regenerating tails have different endogenous currents, we measured the net electrical current flow at tadpole tail stumps following partial amputation. To characterize the electric current profile across the cut tail stump, we measured the current at different positions on the stump. We measured at five positions: (a) dorsal muscle; (b) spinal cord; (c) central muscle/notochord; (d) blood vessel; and (e) ventral muscle (see Fig. 3A). Electric currents soon after amputation were outward (positive value) and similar at all positions (Fig. 3B, day 0). Subsequently, in stage 40 (regenerative) tadpoles, the current at all positions reversed to become inward (negative value in Fig. 3C). These inward currents persisted throughout the whole process of regeneration in stage 40 tadpoles. The current at all positions of stage 45 tadpoles (non-regenerative) stayed outwards (Fig. 3C).

**Table 1**
Electric current (μA/cm$^2$) in unwounded, wounded (time zero) and wounded (day 5) stages 40 and 45 tadpoles.

| Position | Head | Belly | Back | Flank | Tail (side) |
|---|---|---|---|---|---|
| Stage 40 unwounded | −0.27 ± 0.08 | −0.05 ± 0.03 | −0.06 ± 0.02 | −0.06 ± 0.01 | −0.04 ± 0.01 |
| Stage 45 unwounded | −0.16 ± 0.02 ($P>0.17$) | −0.09 ± 0.02 ($P>0.21$) | −0.07 ± 0.01 ($P>0.77$) | −0.11 ± 0.05 ($P>0.24$) | −0.04 ± 0.01 ($P>0.77$) |
| Stage 40 wound zero | 0.81 ± 0.18 | 0.85 ± 0.13 | 0.94 ± 0.2 | 0.59 ± 0.19 | 0.85 ± 0.13 |
| Stage 45 wound zero | 0.12 ± 0.05 ($P>0.11$) | 0.81 ± 0.15 ($P>0.83$) | 1.09 ± 0.05 ($P>0.45$) | 0.93 ± 0.13 ($P>0.14$) | 0.83 ± 0.01 ($P>0.9$) |
| Stage 40 wound day 5 | −0.19 ± 0.06 | −0.12 ± 0.03 | −0.1 ± 0.01 | −0.07 ± 0.03 | −0.04 ± 0.01 |
| Stage 45 wound day 5 | −0.11 ± 0.05 ($P>0.26$) | −0.07 ± 0.06 ($P>0.36$) | −0.17 ± 0.06 ($P>0.2$) | −0.04 ± 0.02 ($P>0.47$) | −0.05 ± 0.01 ($P>0.6$) |

Positive value: outward current, negative: inward. *P* values in rows 2, 4 and 6 show statistical difference (Student's *t* test) between stage 40 and 45 (unwounded, wound time zero, wound day 5) ($n=10$ of each stage from 3 different batches).

We next established a time course of stump current change, measured at position "b" (spinal cord) as shown in Fig. 4B. Before cutting, tails (at the tip) had a very small inward current (less than 0.01 μA/cm$^2$). Immediately after cutting, both stages 40 and 45 tail stumps showed large outward currents (positive value; Fig. 4C, 0 h) which were not significantly different. These initial outward currents diminished during the first few hours. About 6 h after cutting, the regenerative stumps (stage 40) started to show striking inward currents while the non-regenerative stumps (stage 45) maintained outward currents. The current in regenerating and non-regenerating tails became significantly different at 24 h (Fig. 4C). This difference in current (stage 40 inward; stage 45 outward) was apparent from days 1 to 6, and the biggest difference was at 5 days after cutting (Fig. 4D). Interestingly, 5 days is around the time that stage 40 tails begin to regenerate (see Fig. 1B), and also the time when other morphological signs of regeneration appear (see Fig. 7, and section "Possible electrogenic activities at the stump" below). These inward currents persisted throughout the whole process of regeneration in stage 40 tadpoles.

After refractory stage 45 during which most tails fail to regenerate, tail regeneration was restored. We measured the tail current 5 days after cutting at stages 40, 45 and 48 and found that only refractory stage 45 had an outward current, while regenerating stages 40 and 48 had an inward current (Fig. 4E). Tail stump currents therefore correlate well with the ability to regenerate a tail.

### *Tadpole wound electric currents*

In order to determine whether the reversal of the tail stump current was specific for regeneration, or just a stage-dependent epiphenomenon, we measured wound electric currents at different body positions (head, back, flank, belly, side of tail) in stages 40 and 45 tadpoles at time zero (immediately after wounding) and after 5 days ($n=10$ of each stage from 3 different batches). At time zero all wounds had a large outward current and there was no significant difference between stages 40 and 45 at any position ($P=0.17$–$0.78$; Table 1). These large outward currents persisted for 2 h and then diminished, presumably as damaged cell membranes and tissues were repaired. After 5 days, currents at the wound sites had diminished to very small inward currents similar to those in unwounded tadpoles. Again, there was no significant difference between stages 40 and 45 at any position ($P=0.11$–$0.9$; Table 1). There was also no statistical difference between unwounded currents and wound day 5 currents in stage 40 or stage 45 ($P=0.11$–$0.69$), suggesting that the wounds had healed completely.

Thus: (1) the lack of tail regeneration in stage 45 tadpoles is not due to impaired wound healing, and (2) the difference in electric current seen in regenerating vs. non-regenerating tails appears to be a regeneration-specific phenomenon, rather than a general wound effect.

### *Manipulation of stump currents with ion substitution significantly affects regeneration*

To investigate whether altering tail currents could change regeneration rate and/or percentage regeneration, we substituted ions that may be involved in generating the endogenous ionic currents (Na, Cl) and measured tail current and regeneration. We have previously shown a strong correlation between cornea wound electric current and cornea wound healing rate (Reid et al., 2005). Tadpoles were incubated in Na- or Cl-free solution (see methods) before and after cutting. Tadpoles (stage 40) in Cl-free solution had normal tail currents 5 days after cutting ($n=14$ from 3 different batches), and showed normal regeneration rates ($n=23$ from 4 different batches) (Fig. 5A, B). Tadpoles in Na-free solution had significantly reduced currents ($P<0.01$) ($n=17$ from 3 different batches), and correspondingly reduced regeneration rates ($P<0.03$) ($n=20$ from 4 different batches). Similarly, percentage regeneration (normally 77.6%) was dramatically reduced in Na-free solution (47.2%; $P<0.01$) ($n=27$ from 4 different batches), but not significantly changed in Cl-free solution (76%; $P>0.8$) ($n=31$ from 4 different batches).

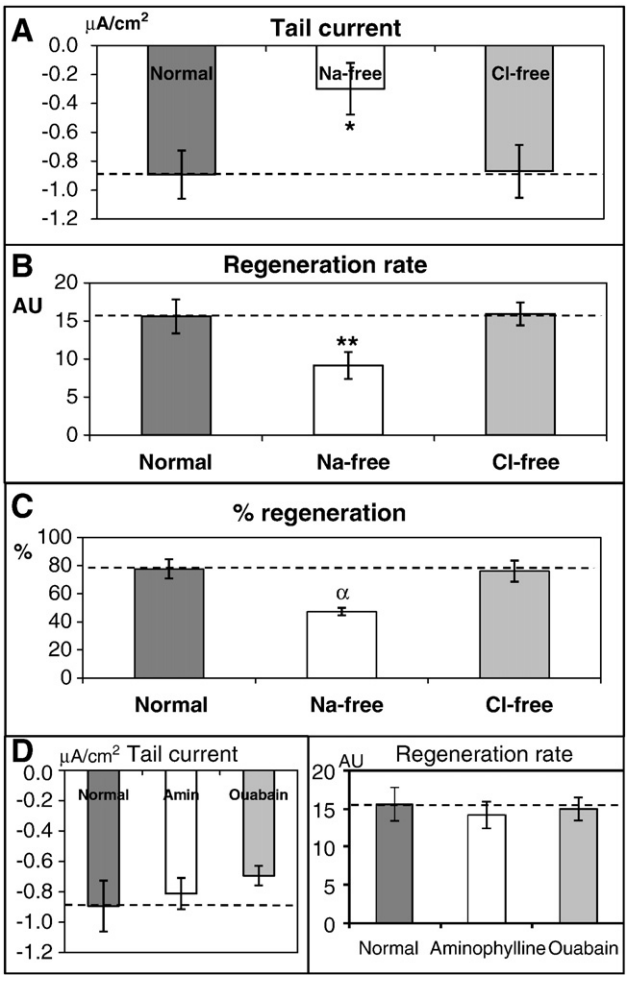


**Fig. 5.** Sodium in the bathing solution is important for stump currents and tail regeneration. Tadpoles (stage 40) were incubated in solution lacking sodium. (A) In sodium-free solution, the stage 40, day 5 tail current was significantly reduced (*$P<0.01$) to 33% of control ((−0.299/−0.893)*100). Tail current in chloride-free solution was unchanged. "Normal" data taken from Fig. 4D (day 5). (B) Regeneration rate ("normal" $n=25$ from 4 different batches) was also significantly reduced in sodium-free solution (**$P<0.03$) but unchanged in chloride-free solution (AU = arbitrary units). (C) The percentage of tails that regenerated (stage 40) was significantly less in sodium-free solution ($^{\alpha}P<0.01$), but unchanged in chloride-free solution. "Normal" data taken from Fig. 1C. (D) Drugs that alter ion transport in mammalian epithelia did not affect stump currents or regeneration. Tadpoles (stage 40) were incubated in drugs which increase (aminophylline) or decrease (ouabain) ion pumping, and are known to alter wound current and wound healing in rat cornea. Neither drug had any effect on tail current, regeneration rate or % regeneration (data not shown).

In Na-free solution, stage 40 tadpole tail currents at time zero (soon after cutting) were smaller than in normal solution ($0.95\pm0.18$ μA/cm$^2$, $P<0.002$) ($n=9$ from 3 different batches). This was the case only if tadpoles were pre-incubated for a few hours (e.g. overnight) in Na-free solution. Tadpoles taken from normal solution into Na-free, then anesthetized, tail cut and measured immediately had larger currents ($3.17\pm0.52$ μA/cm$^2$; $P<0.01$) ($n=8$ from 3 different batches). This suggests that sodium is a major component of the initial large outward currents.

To exclude the possibility that Na-free solution may adversely affect wound healing, and in turn reduce tail regeneration, we studied the effect of Na-free bathing solution on small fin-punch wounds. We

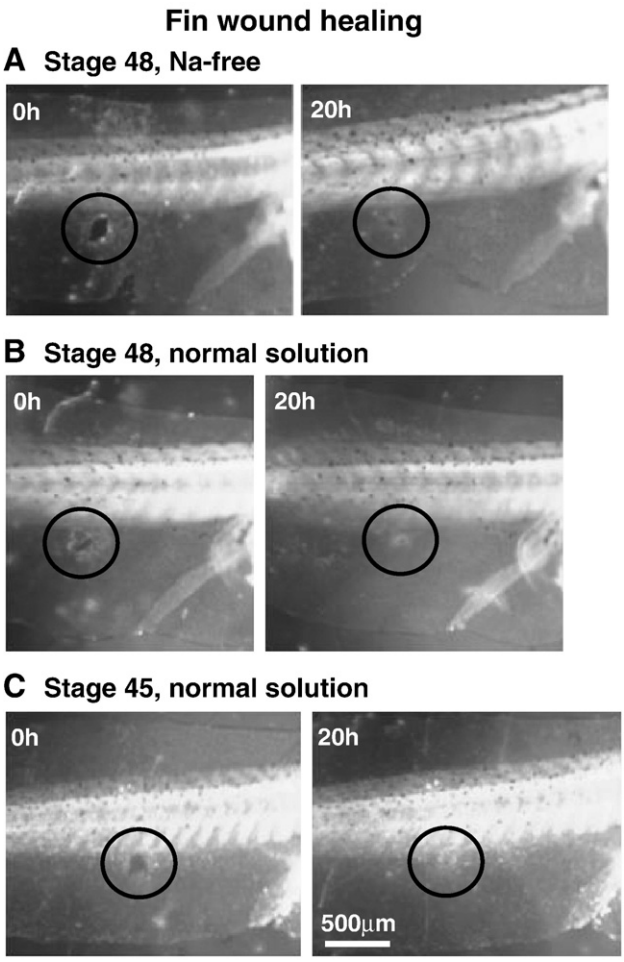


**Fig. 6.** Sodium and wound healing. (A, B) Fin punch wounds (stage 48) healed normally in Na-free solution (both Na-free and Normal $n=8$ from 2 different batches). (C) Punch-wounds in fin also healed normally at stage 45 ($n=12$ from 2 different batches). Scale bar 500 μm.

found that small (200–300 μm diameter) fin-punch wounds healed at the same rate whether in normal or Na-free solution, healing almost completely in 20 h at stage 48 (Fig. 6A, B). Fin wounds have very small currents, even immediately after wounding ($0.28 \pm 0.08$ μA/cm$^2$, compared with $4.38 \pm 0.17$ μA/cm$^2$ in cut tails), probably too small to influence cell migration, which may account for the lack of inhibition of fin wound healing in Na-free solution. Thus, the reduction of tail re-growth in Na-free solution is likely to be a regeneration-specific effect, not just an inhibition of wound healing. Interestingly, 91% of stage 45 tadpoles showed normal fin punch healing (Fig. 6C), suggesting that the absence of tail re-growth at this stage is also regeneration-specific rather than a general inhibition of wound healing.

*Drug treatments demonstrate different ionic transport mechanisms from mammalian epithelium*

To determine whether we could stimulate or inhibit regeneration by manipulation of the tail current pharmacologically, we used various drugs which we have previously shown increase (aminophylline, ascorbic acid) or decrease (ouabain, furosemide) mammalian cornea wound electric current and healing rate (Song et al., 2002; Reid et al., 2005). Aminophylline enhances chloride efflux in frog cornea (Zadunaisky et al., 1973), ascorbic acid increases sodium and chloride transport across amphibian cornea (McGahan and Bentley, 1982), ouabain blocks the Na/K ATPase in rabbit cornea (Wigham et al., 1994), and furosemide inhibits the sodium/ potassium/ chloride co-transport system in frog cornea (Patarca et al., 1983). Incubating tadpoles in these drugs had no effect on tail stump current, regeneration rate, or percentage regeneration, suggesting that amphibian tail stump currents are generated by different ion channels/pumps than in mammals (Fig. 5D; percentage regeneration data not shown; data for furosemide not shown; tadpoles in ascorbic acid died prematurely).

*Possible electrogenic activities at the stump*

To explore the possible mechanisms of the generation of electric currents at regenerative stumps, we studied the structure of tail stump epithelium, which is one of the most electrogenic tissues due to its ability to maintain a transepithelial potential. We also examined cell membrane potentials using a membrane potential-sensitive dye DiBAC$_4$(3). We noticed anatomical differences between regenerating and non-regenerative tails. All non-regenerating tail stumps (irrespective of stage) were quickly "sealed over" by a smooth, thick "skin like" epithelium (Fig. 7C), whereas regenerating tail stumps formed a thinner epithelium, which does not have a very clear boundary and

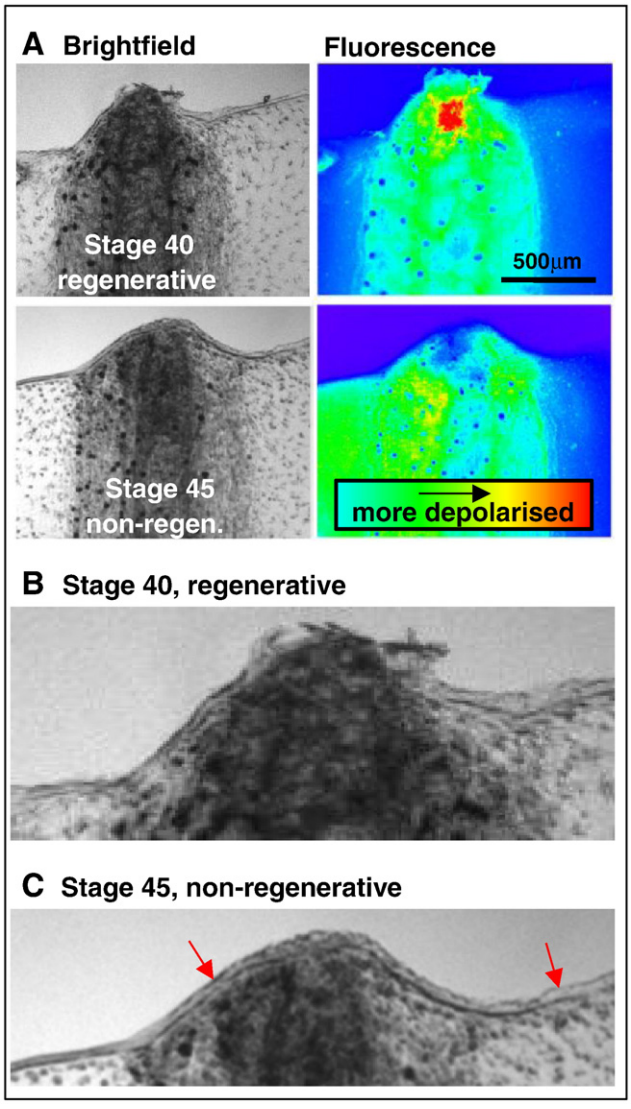


**Fig. 7.** Possible roles of depolarized cells and epithelial morphology. (A) Using the membrane potential-sensitive fluorescent dye DiBAC$_4$(3), aggregates of highly-depolarized cells were observed in regenerating (stage 40) tails 5 days after cutting. Non-regenerating tails had no such aggregates, but had a thick skin-like epithelial membrane covering the cut stump (arrows in C) which was not present in stage 40 tails (B). Scale bar 500 μm.

is less smooth (Fig. 7B). Similar results have been described in Beck et al. (2003).

$DiBAC_4(3)$ staining showed that regenerating tail stumps developed, during the first few days, an aggregate of highly depolarised cells (Fig. 7A), which appeared to be the nucleus of regeneration, beginning around five days. This aggregate of highly depolarized cells was absent in non-regenerative stumps. Similar results were reported by the Levin laboratory (Adams et al., 2007). Thus, in stage 40 and stage 45 tadpoles, there are distinctive epithelial morphological differences between regenerating and non-regenerating tails, and an aggregate of depolarized cells present only in regenerating tails.

## Discussion

Biophysical factors are emerging as a very powerful control mechanism in regeneration and wound healing. We sought to test the hypothesis that electric currents play a role in regeneration using the tail regeneration model of *X. laevis* tadpoles. We found that stage 40 *Xenopus* tadpole tails began to regenerate about five days after cutting, whereas stage 45 tadpoles have lost this regenerative ability. Loss of regeneration correlated very well with loss of a unique reversal phase of naturally-occurring electric currents at the amputated tail stumps. Decreasing the reversed currents significantly decreased regeneration in stage 40 tadpoles. The reversal of electric current direction may involve mechanisms other than regular electrogenic epithelial transportation of ions.

### *Electric currents at amputation stumps predict tail regeneration*

Endogenous electric fields, produced by ionic currents, have been implicated in the control of regeneration and development (reviewed by Borgens, 1983; Nuccitelli, 1988; Levin, 2003; Robinson and Messerli, 2003; McCaig et al., 2005). Application of an electric current of ~10 μA increases regeneration in injured spinal cord in lamprey and guinea pig (Borgens et al., 1986, 1987, 1990). These data have led to clinical trials in human subjects (Shapiro et al., 2005). Our results on the time course and spatial distribution of electric currents at amputated tadpole tails show for the first time that there is a unique pattern of current flow reversal in regenerating tails. This reversal distinguishes the regenerative and non-regenerative tails—those that will regenerate have the signature of current reversal, whereas those which will not regenerate do not have this reversal phase (see Figs. 3, 4). Significantly, decreasing the electric current in the reversal phase reduced regeneration (Fig. 5). It is therefore highly probable that the electric currents are a complementary and powerful controller of regeneration. Adams et al. (2007) have demonstrated that proton pump-dependent changes in membrane voltage are necessary as well as sufficient for tail regeneration. Whether this reversal of current is sufficient alone to induce regeneration is yet to be tested.

### *The inward currents appear to be involved in regeneration*

What happens if we alter this electrical activity, can we alter tail regeneration? We placed tadpoles in solutions lacking ions known to be important in epithelial wound healing in cornea (Na, Cl; Reid et al., 2005). Tadpoles in Cl-free solution had normal tail stump electric currents and normal tail regeneration. However, tadpoles in Na-free solution had significantly reduced tail stump currents, and correspondingly reduced tail regeneration rate and percentage regeneration (Fig. 5). We have found that sodium is a major component of the endogenous ionic/electrical current in rat cornea wounds (Reid et al., 2005) and it has been shown that wound healing in *Xenopus* neurulae (oocytes) is inhibited in sodium-free solution or by drugs which inhibit sodium flux (Rajnicek et al., 1988). Sodium is probably only one ion contributing to the endogenous electric current, others being potassium, calcium, etc. The results in Fig. 5A suggest that sodium contributes about two thirds of the ionic content of the tail current (percentage current in Na-free solution $= (-0.299/-0.893) \times 100 = 33.48\%$ of normal).

### *Electric currents at amputation stumps are likely to be actively regulated*

The dynamic course of stump currents strongly suggests active regulation of electric currents at the stump. We found that tail stumps (and wounds at other positions) initially had large outward currents. The reversal of the stump currents was an unexpected discovery. This is because normally the transepithelial potential generates outward currents upon injury. Active transport of $Na^+$ by the epithelium generates higher electric potential at the basal side (inside). Damaging the epithelium forms a short-circuit, and the transepithelial potential difference drives current flow out of the wound. Such outward wound electric currents/fields have been measured with many techniques - micro-glass electrodes, vibrating probe, micro-electrode arrays and bioelectric imager (see review by Zhao, 2009). Therefore, the accepted concept of wound electric currents driven by the epithelium does not explain the inward current in the regenerating stage 40 tails. This reversal of stump current is likely to be regulated differently from wound electric currents.

### *Regenerative epithelium and aggregate of depolarized cells*

The mechanisms that cause the reversal of the stump electric current direction are not known. Two things may contribute to the regulation of stump currents: the electrogenic epithelium and cell polarization. The differences between regenerating and non-regenerative stumps have been described in detail by the Slack and Levin laboratories (Beck et al., 2003; Adams et al., 2007). Epithelial morphology in stage 40 regenerating tails and stage 45 non-regenerative tails are very different (Fig. 7). The thick, smooth, skin-like epithelium with well defined structure in stage 45 stage tadpoles might function to restore normal skin function and form a tight barrier, indicated by very small electric currents at the stumps. In contrast, the epithelium on the regenerating stage 40 stumps showed uneven and sometimes broken morphology.

The aggregate of depolarized cells may play some role in the current reversal. The initial nucleus of tail regeneration appears to be this clump of cells in the centre (midline) of the tail of stage 40 tadpoles (Fig. 7, and Adams et al., 2007). In most stage 45 tails, however, there was no aggregate of depolarised cells, and regeneration did not occur. This aggregate of depolarised cells may be a "regeneration bud" of undifferentiated cells which are capable of differentiating into various cell types.

Formation of distinct epithelia and aggregation of cells occurs in early embryos, and also in regenerating tissue/organs. When a salamander limb is amputated a layer of epidermis covers the stump surface. During the first few days after injury, this so-called "wound epithelium" transforms into a layer of signaling cells called the apical epithelial cap, which has a vital role in regeneration. Fibroblasts from the connective tissue migrate across the amputation surface to meet at the center of the stump. They multiply to form a blastema which is the progenitor of the new limb. A similar process appears to occurs in *Xenopus* tadpole tails, but not at stage 45 where a thick skin-like epithelium forms over the cut surface, rather than a thin "wound epithelium". This early difference in response to amputation may be responsible for the lack of regeneration at this stage, perhaps via changes in the tail stump electric field, which has dramatic effects on cell migration during wound healing, regeneration, and other cellular behaviors (Zhao et al., 2006).

Finger regeneration after accidental amputation in children depends on how the stump is treated. Sewing over a flap of skin prevents re-growth, whereas if the stump is left open and moist, the finger tip regenerates perfectly (Illingworth, 1974; Illingworth and

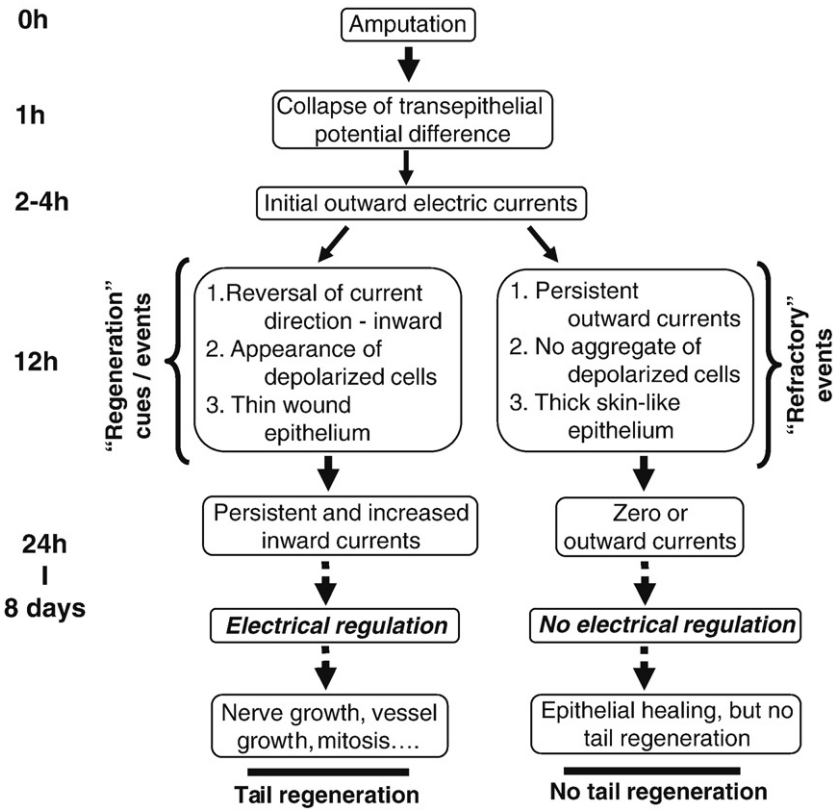


**Fig. 8.** Schematic model depicting the possible role(s) of electrical events during tail regeneration. Amputation breaks the epithelial barrier, collapses the transepithelial potential difference, and induces large outward currents. The outward electric currents last only a few hours in the tails that regenerate. A sequence of cellular events that may regulate electric current flow occur 4–24 h after amputation and persist for up to a few days. These events may include expression of the V-ATPase that drives induction of NaV1.2 and KCNK1 (Adams et al., 2007). The epithelium covering regenerating stumps was rough and had no visible basement layer under phase optics. In contrast, non-regenerating stumps quickly (1–2 days) grew a thick, smooth, skin-like epithelium over the cut stump. In regenerating tails, a group of highly depolarized cells appeared under the site of tail re-growth. These cells might cause the electric current in the regenerating stump to reverse direction and became inward. Altering the inward currents decreased regeneration. In contrast, tails during refractory stages did not reverse the current direction, which appeared to contribute to the inability to regenerate.

Barker, 1980). These results suggest that blocking stump currents prevents regeneration. Significant molecular insights have also been gained using genetic manipulation. A proton pump, the vacuolar-type adenosine triphosphatase (V-ATPase), is necessary and sufficient for *Xenopus* tadpole tail regeneration (Adams et al., 2007). Thus, regulated expression of ion transporters contributes to ion fluxes and electric currents at the stump.

We have previously shown that pharmacological drugs which alter ion flow (and therefore wound electric field) also alter wound healing rate in rat cornea (Reid et al., 2005). We placed tadpoles in drugs which enhance (aminophylline, ascorbic scid) or decrease (ouabain, furosemide) ion flow. There was no significant effect on tail current or regeneration (Fig. 5D). Adams et al. (2007) previously showed that ouabain had no effect on tadpole tail regeneration. This suggests that ion pumping and wound current generation in amphibians occurs by different mechanisms than in mammals, e.g. by ion pumps/channels that are insensitive to these drugs.

*Electrical events in tail regeneration*

We propose a model to describe the possible effects of electrical activity on tail regeneration (Fig. 8). Amputation breaks the epithelial barrier and induces large outward currents. A few hours later, the electric current reverses direction at tails that will regenerate. A series of cellular events that may regulate (or occur in parallel with) the electric currents come into play and persist for up to a few days. This electrical regulation may stimulate and guide nerve growth, blood vessel formation and cell proliferation, migration and differentiation, culminating in tail regeneration. In contrast, tadpoles during refractory stages do not reverse the current direction, which appears to contribute to their inability to regenerate.

In conclusion, we have found a strong correlation between electric current direction and regeneration in amputated tadpole tails. At the crucial point as regeneration begins (day 5 post amputation) tails which regenerated had inward currents whereas tails which did not regenerate had outward currents. Since an electric field directs the rate and direction of *Xenopus* neurite growth *in vitro* (McCaig and Zhao, 1997; Rajnicek et al., 1998; McCaig et al., 2002), the electric signal might be one of the key players controlling regeneration. Manipulation of the electric signal significantly altered regenerative ability. We propose that post-amputation, electric currents at the stump are a key "regeneration factor" in tail/spinal cord regeneration which may participate in or act in parallel with BMP-Notch-mediated regeneration.

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